METRONIDAZOLE EFFECTS ON HINDGUT MICROFLORA RESIDING WITHIN THE SUBTERRANEAN TERMITE *RETICULITERMES FLAVIPES*

A Thesis

Presented to

the Faculty of the College of Science and Technology

Morehead State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

By

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1 May 2013

Accepted by the faculty of the College of Science and Technology
Morehead State University, in partial fulfillment of the requirements for the
Master of Science degree.

Director of Thesis

Master's Committee: _____, Chair

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Date

METRONIDAZOLE EFFECTS ON HINDGUT MICROFLORA RESIDING WITHIN THE SUBTERRANEAN TERMITE *RETICULITERMES FLAVIPES*

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The termite gut is a well-known existing micro ecosystem that involves the combined efforts of flagellates (protozoa) and bacteria for energy metabolism of the host. The termite host and their microbiota have coevolved into a form of commensal mutualism.

Reticulitermes flavipes was chosen for this study because of their economic importance, unique control challenge behavior, and the possibility in holding the vital component in solving the energy crisis the world will face in the near future.

Furthermore, *R. flavipes* are easy to maintain in the lab and are abundant in the Kentucky area, thus are easy to obtain.

The first objective of this study was to determine if, and what effects, heat and metronidazole have of the symbiotic microflora of *R. flavipes*. By characterizing the effect of heat and metronidazole on the microflora, a symbiotic relationship may be established to show the importance of prokaryotic residents in maintaining a fit environment for flagellates or vice versa. The second objective was to measure the mortality rate differences between experimental and control groups. Comparisons of eukaryotic content and consumption yielded increased mortality among groups treated with metronidazole. In addition, heat intensified the affects of metronidazole slightly leading to higher mortalities within metronidazole-treated groups.

Future studies identifying the prokaryotic and eukaryotic species affected by metronidazole application will lead to a better understanding of mortality in treated termites.

Accepted by: _____, Chair

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Acknowledgements

I am very thankful for the opportunity to conduct this experiment and for the funding provided by the Morehead State University Biology and Chemistry Department to do so. I am extremely grateful for Dr. Geoffrey Gearner and his guidance and encouragement in helping to complete this project. I would also like to thank Dr. Sean O'Keefe for his help with the literature and entomological applications within this experiment. I am also in debt to Dr. David Smith who helped in the statistical summation as well as assistance in providing me with laboratory supplies. Additionally, I would like to thank the University of Kentucky Department of Entomology and Dr. Xugou Zhou for his assistance in the design of this project. Furthermore, I would like to extend my appreciation toward Li Tian in helping with the collection of termites and for his time in showing me maintenance and care of the termites.

Lastly, I would like to thank Matthew Nail, my family, and friends for their support and reassurance during both the hard and happy times that come with all research projects.

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Introduction and Literature Review

1.1 Anatomical and Social Organization of Termites

Termites are social, terrestrial insects that inhabit nearly two-thirds of Earth's land between 45^0N and 45^0S latitude, and they are also present in temperate zones (Stingl, 2004). Termites are included within the superorder Dictyoptera that also includes cockroaches and mantids. Specifically, termites belong to the order Isoptera. This order can be divided into lower and higher termite groups based on social structure and anatomy. The lower termites are organized into six families: *Mastotermitidae, Kalotermitidae, Hodotermitidae, Rhinotermitidae, Serritermitidae,* and *Termopsidae* (Edwards, 1986). The higher termites are placed in one family, *Termitidae* (Figure 1). There are over 2,600 termite species, and there may be 500-1,000 species yet to be discovered.

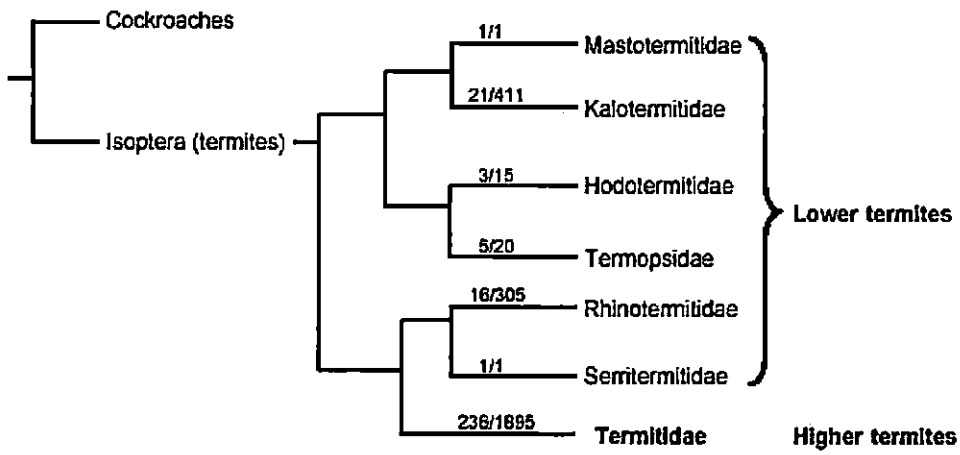


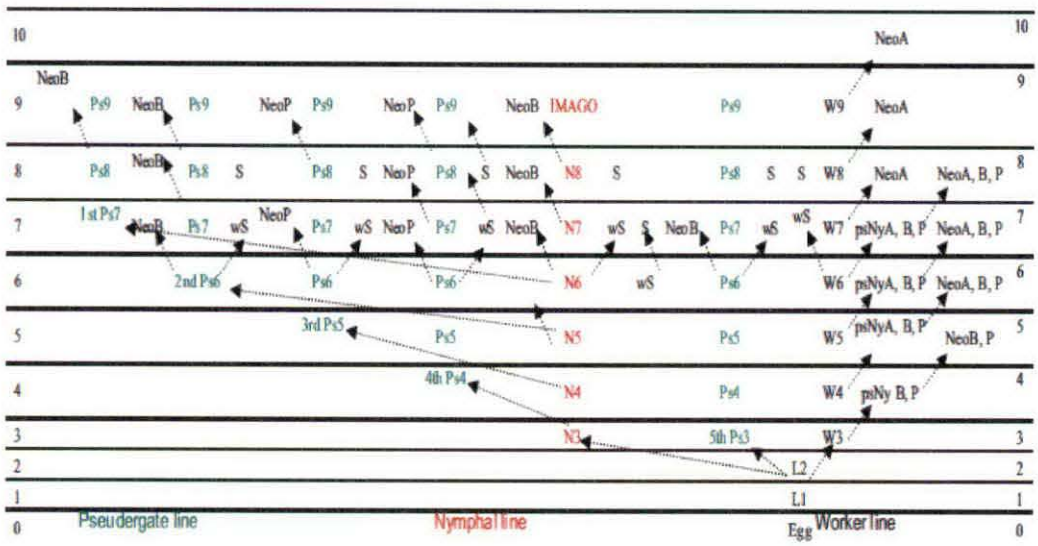
Figure 1. Phylogenetic representation of termites and relatives. Numbers represent genera/species present within each family (from Stingl, 2004).

Termites are generally found in the tropical and temperate areas of the world and occupy about seventy percent of the land (Edwards, 1986). Lower temperatures used to be responsible for the absence of termites, but because of the construction of centrally heated buildings, termites are now able to colonize colder areas in the world (Edwards, 1986). Those involved in pest control usually group termites into three main categories before looking specifically at family classification. The three main categories include: subterranean, dampwood, and drywood termites. Subterranean termites belonging to the family *Rhinotermitidae*, are the most widely distributed family of termites, occurring throughout the tropical, subtropical, and temperate regions of the world (Vargo, 2009). Subterranean termites build their nests in the soil and rely primarily on the soil for moisture.

Termite colonies live inside a nest, and if the nest is covered with, or made from earth to create a structure protruding above the soil surface, then it becomes a mound. Nests and mounds have many functions including: protection against the environment, keeping internal climates constant, and fortification against predators (Eggleton, 2011). Termites build their nests with their feces making them less vulnerable to pathogens, cheap to produce, and structurally sound.

The most important feature of termites that separates them most from other insects is their communal nest behavior (Edwards, 1986). Termites are defined as social insects because of the different members' dependence on one another. Because they exhibit social behavior, termites have developed a caste system. The caste of each individual can be determined throughout post-embryonic development,

contingent on the various pheromones present in the colony (Janowiecki, 2012). Overall, the colony has immatures as well as three main adult castes that contain: reproductives (queens, kings, alates), workers, and soldiers (Eggleton, 2011). Many studies have been conducted reviewing the developmental pathways of *Reticulitermes flavipes* in an attempt to better understand how to combat pest colonies (Figure 2).



L = Larve; N = Nymphs; Neo = Neotenic Reproductives (a = apterous; B = brachypterous; P = pseudapterous); PsNy = Pseudonymphs (A; B; P); Ps = Pseudergate ("false worker"); S = soldier; wS = white(pre-) soldier; W = worker

Figure 2. Developmental pathways of Reticulitermes flavipes in laboratory settings (adapted from Hu and Forschler, 2012).

Each member of the separate castes has their own tasks. In addition, castes can be physically distinguished by several key traits. Workers of the lower termites usually lack pigment or are lighter in color compared to other caste types. There is also no sign of wing development or eye structures. In contrast, soldiers have an enlarged and sclerotized head capsule, mandibles which aid in colony defense, and

are pigmented (Janowiecki, 2012). The reproductive caste has multiple forms, but the distinguishing feature is wings in the primary alates. Soldiers have the sole job of protecting the colony, whereas the king and queen mate regularly and produce eggs for the colony (Eggleton, 2011). Alates eventually will leave the nest and pair to start new colonies. In order to colonize new nests, swarm events occur where winged reproductive termites disperse to initiate new colonies (Janowiecki, 2012). In these swarms, both male and female winged adults usually disperse concurrently from the colony and pair to start incipient colonies away from their colony of origin.

The termite life cycle commences with the dispersion of winged alates to colonize new resources. The alates pair, shed their wings, and establish a new nest (Janowiecki, 2012). The castes are determined during post-embryonic development due to pheromones present, with each larva (first or second instar termite) capable of developing into a worker, soldier, or reproductive. When a termite first hatches from its egg, it is termed a first instar. After shedding of its exoskeleton for the first time, the first instar is termed a second instar. The termite can go through up to seven molting stages.

Total time of development from egg to adult can take up to seven weeks. Nymphs are the first developmental stage after the egg and are wingless. Worker termites are the first to be produced followed by production of three types of nymphs: pseudergates or false workers that molt continuously, nymphs with wing pads that develop into winged male and female reproductives; and soldier nymphs.

Primary reproductives come from nymphs with wing pads and are named

alates. These eventually become the king and queeno of the newly founded colonies. A neotenic reproductive is a reproductive that is not derived from an alate and still retains some juvenile characteristics (pseudonymph). Secondary, or nymphoid reproductives, are neotenics derived from nymphs and are brachypterous (rudimentary wings present). These have wing pads and their body pigmentation is generally a yellow hue. Tertiary, or ergatoid reproductives, are derived from workers and are usually smaller than nymphoid reproductives and are apterous (no wing pads present) containing very light pigmentation (Janowiecki, 2012). Figure 2 compiles the many adjacent pathways that a colony member can undertake that ensures the success and continued perpetuation of a colony. In summary, there are many reserved forms other than nymphs with wing pads that can develop into a reproductive. However, wingless reproductive colonies will eventually die off due to resource shortages.

Workers in the colony have many jobs including: foraging for new food and water, building and repairing of colony structures, and tending to the immature, alates, and king and queen. Worker importance can be clearly understood by looking at the integrated processes used in termite feeding. The workers of colonies tend to the immature instars that are essentially helpless (Eggleton, 2011). In lower termites, workers will assimilate food and pass nutrients as well as some flagellates to the immature either by proctodeal trophallaxis or stomodeal trophallaxis. Proctodeal trophallaxis involves feeding by secretions from the anus, whereas stomodeal trophallaxis includes feeding from the glands in the head and mouth (Eggleton, 2011).

Proctodeal feeding is also shared by members of the colony because of the loss of symbionts due to molting (Rust and Nan-Yao, 2012). Overall, the highly developed social system of termites has allowed for increased developments in adaptive radiation and feeding habits (Tayasu, 1998).

Termites are considered pests when they damage products of value to humans (Edwards and Mill, 1986). Food sources that termite species are most likely to consume are listed in Table 1.

Table 1. Termite material for consumption (from Edward and Mill, 1986).

Dead wood (structural timbers, furniture, transmission poles, dead trees).
Rotten or decaying wood (structural timbers, poles, trees).
Living wood (shrubs and trees)
Fungi (either outside or specifically grown inside the nest).
Grass and herbs (including many crop plants).
Plant debris (dried grass and twigs, old birds' nests).
Soil (containing organic matter-humus).
Animal dung (containing plant remnants).
Stored grain and other human food.
Paper and books.
Plant roots which invade the nest.
Non-vegetable materials used by man (e.g. underground cable insulation).

Many species of lower termites feed almost exclusively on wood (lignocelluloses), although this food is difficult to digest because the wood fibers are composed of stable polymers of cellulose (La Fage and Nutting, 1978). Most termites prefer wood that has been attacked by fungi, which makes it easier to utilize due to

partial decomposition. Fungi degraded wood is also often richer in protein because of fungal mycelia that can also be digested or removed by termites prior to the meal (Radek, 1999). Rhinotermids prefer to feed on dead wood and grass and have relatively simple guts (Bignell, 2011). Early termites gained the ability to digest cellulose by acquiring cellulose-producing symbiotic protozoans (flagellates). These protozoa are named symbiotic because of their integrated relationships they share with prokaryotes during the digestive process of the termite.

The termite digestive system is split into three distinct parts: foregut (stomatodeum), midgut (mesenteron), and hindgut (proctodeum) (Eggleton, 2011). Flagellates are responsible for the fermentation of the partially digested food under anaerobic conditions in the hindgut of the termite. Overall, the digestion process is divided between the mid-gut cellulase production and hindgut symbiotic microbial fermentation. *Termitidae* members, in contrast to lower termites, have selectively lost their hindgut flagellates due to beneficial adaptations for a more efficient digestion of wood, grass, soil, and humus and rely only on digestive enzymes such as cellulase to acquire nutrients (Eggleton, 2011). However, all termite families still produce an endogenous cellulase in their midgut regions.

1.2 Flagellate Population within the Termite Hindgut

The termite gut is a complex microecosystem consisting of a diverse array of flagellates, bacteria, archae, and fungi. The relationship between the termite host and most of its gut symbionts is a form of obligatory mutualism (Eggleton, 2011). Each termite species hosts several flagellate species that are nutritionally specialized and aid in the digestion of lignocelluloses in their specific habitat (Brune and Ohkuma, 2011). Moreover, the flagellate population within the hindgut of termites makes up more than one-third of the body weight of the termite (Stingl, 2004).

In 1856, Lespes first mentioned living cells in the hindgut of termites (Lewis, 2001). The protozoa in the hindgut of termites is now recognized into three orders: Trichomonadida, Oxymonadida, and Hypermastigida. Termite protozoa were first described from *R. flavipes* by Leidy in 1877 (Lewis and Forschler, 2004). There have been a total of eleven species observed in R. flavipes which include: *Dinenympha fimbriata, Holomastigotes elongatum, Microjoenia fallax, Monocercomonas sp. Pyrsonnympha major, Pyrsonnympha vertens, Spironympha kofoidi, Spirotrichonympha flagellata, Trichomitus trypanoides, Trichonympha agilis* and other *Spirotrichonympha spp.* that have not yet been described. Light microscopy can reveal the structure of these eleven species (Figure 3).

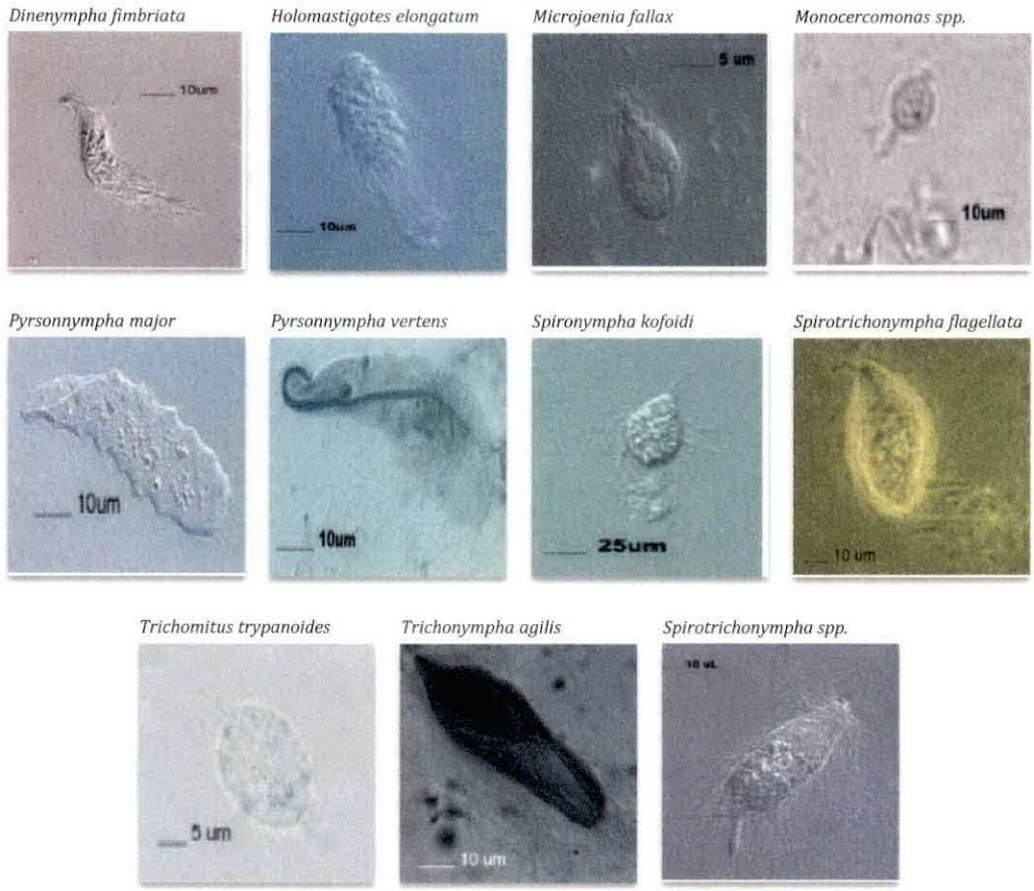


Figure 3. Eleven most common protist species from *Reticulitermes flavipes* taken on 100x magnification (from Lewis, 2001).

In addition, dichotomy can be used to decipher between the different species present (Figure 4). More often than not, the two species *R. flavipes* and *R. virginicus* are hard to discern from one another because of their morphological similarities and

overlapping geographical ranges. However, flagellate identification can help in termite species identification, as *Dinenympha fimbriata* is not found in *R. virginicus.*

1. a. More oval shaped-**GO TO 3**
   b. Not oval shaped-**GO TO 2**

2. a. Fusiform cell shape-**GO TO 5**
   b. Pyriform or lanceolate cell shape-**GO TO 6**

3. a. Small, 4.5 to 30 μm, axostyle posterior, thickened-***T. trypanoides***
   b. Small, 3.5-20 μm, thickened axostyle absent-**GO TO 4**

4. a. 3 anterior flagella, articulated flagella-***Monocercomonas spp.***
   b. More than 8 anterior flagella, undulating membrane-***M. fallax***

5. a. Anterior flagella-***T. agilis***
   b. Posterior flagella-**GO TO 7**

6. a. Large, 75-140 μm in length, undulating membrane-***P. major***
   b. Under 75 μm in length-**GO TO 9**

7. a. Club shaped, 100 to 150 μm in length, large, 4 to 8 flagella that extend posteriorly-***P. vertens***
   b. Not club shaped, covered densely with flagella-**GO TO 8**

8. a. Spiral flagella, length 100-150 μm in length, large-***H. elongatum***
   b. Flagella extending posteriorly, axostyle extending posteriorly-***S. flagellata***

9. a. Pyriform shape, numerous flagella extending posteriorly, 20 to 35μm in length-***S. kofoidi***
   b. Lanceolate form mostly: may be pyriform, but if pyriform, larger than 35 μm usually-**GO TO 10**

10. a. Body flattened, twisted, numerous flagella, 24 to 50 μm in length, lanceolate-***P. gracilis***
    b. Body not flattened, axostyle extending posteriorly, 52 to 64 μm in length-***D. fimbriata***

Figure 4. Dichotomous key describing features unique to each flagellate species present in *Reticulitermes flavipes* (adapted from Lewis and Forschler, 2006).

Primary digestion takes place in a widened hindgut region where wood is phagocytosed by flagellates into vacuoles which leads to maximum absorption for the termite. Fermentative activities of flagellates during digestion of the lignocelluloses result in short-chain fatty acids that can be absorbed by the termite (Brune and Ohkuma, 2011). The vital role of flagellates was illustrated in a set of starvation

experiments conducted previously by Cleaveland (1923), Hungate (1938), and Trager (Hu, 2008). Many of the flagellates residing within the gut have been shown to be sensitive to certain conditions in terms of their ability to digest cellulose. *T. fimbriata* has been shown to be a true cellulolytic, in other words, it can still be found even after many other species of flagellates have been eliminated (Hu, 2008). On the contrary, *P. vertens* has been shown to be a facultative cellulolytic species because of its disappearance after certain cellulolytic species have been eliminated (Lewis and Forschler, 2004). However, studies involving metabolic processes of termite gut flagellates are quite limited due to the inability of obtaining pure cultures for study.

Flagellates inhabiting the hindgut of termites can be quantified using a hemocytometer or can be visualized using a wet mount and saline solution (Lewis 2001).

In addition to the flagellates described in the digestion of cellulose, bacteria also have an important role (Raina, *et al.*, 2004). Most of the flagellates house a multitude of bacterial endosymbionts that have not yet been identified (Stingl, 2004). In addition, ectosymbionts can be attached to the surface of protozoa. The intricate interactions of flagellates and their associated bacteria have been under investigation for quite some time.

1.3 Bacterial Population within the Termite Hindgut

Termites harbor diverse and unique microbial populations in their hindgut at a density of 10^5 to $10^{11} mL^{-1}$ of gut fluid (Brune and Ohkuma, 2011; Waller and La Fage, 1978). Bacteria are also present in the foregut and midgut in lower densities

(Waller and La Fage 1978). Morphological observations, assessments of overall gut metabolism, and measurements of physiochemical conditions in the gut environment, have all contributed to significant progress in understanding the roles of gut microbial community and host nutrition (Brune and Ohkuma, 2011). Molecular approaches in the past couple of decades have also enhanced our ability to assess natural microbial communities and have been applied for understanding the diversity, structure, and evolution of microbiota in termite guts (Brune and Ohkuma, 2011). Moreover, 16sRNA sequencing has also shed light on the diversity of bacteria in termite gut communities. Bacterial 16sRNA genes extracted from the gut homogenates can be amplified using PCR and universal primers. The PCR products can then be cloned, sequenced, and sorted into groups (Brune and Ohkuma, 2011). Figure 5 shows the phylogenetic tree derived from 16sRNA gene sequences of hindgut bacteria residing within *R. flavipes*.

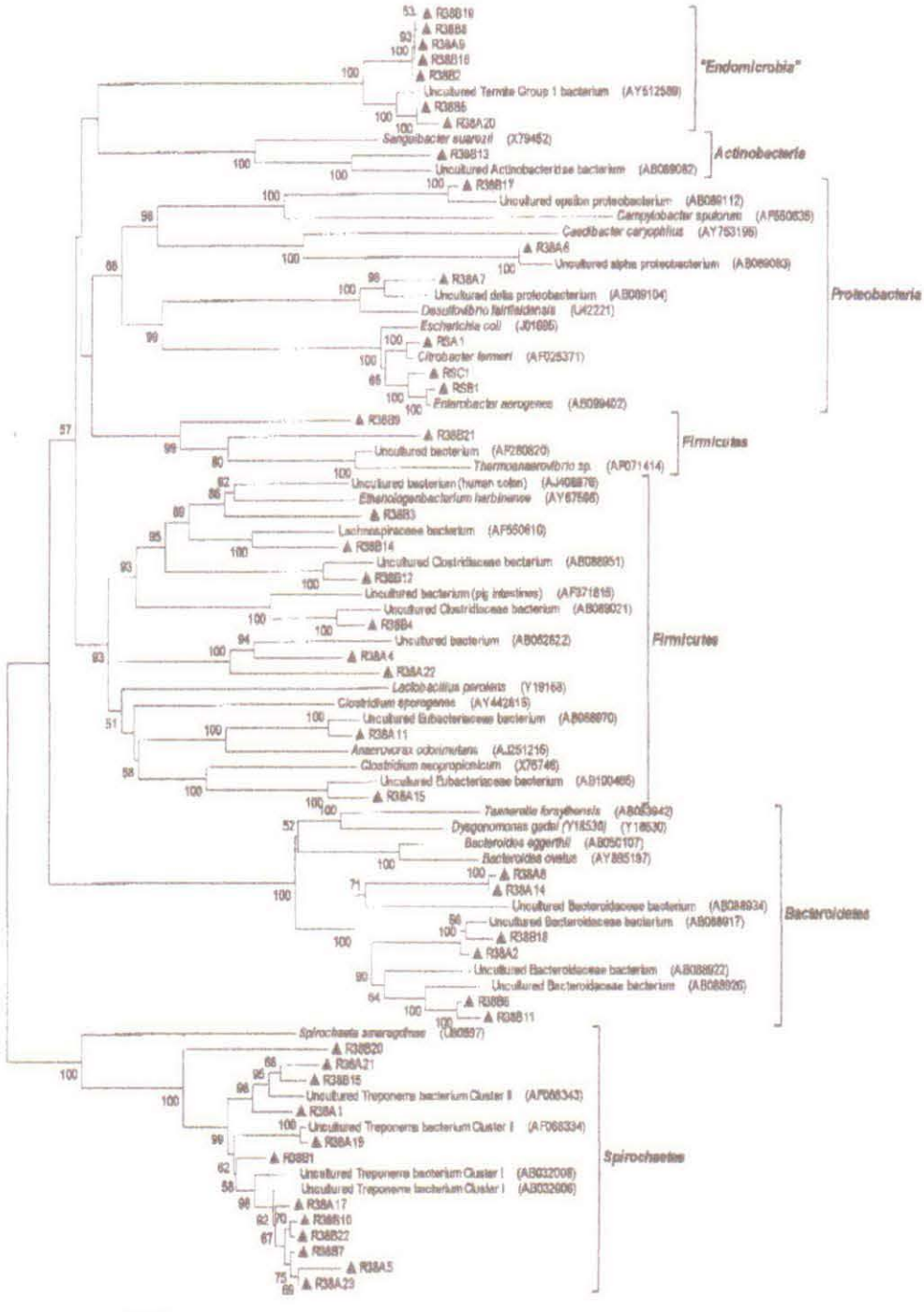


Figure 5. Phylogenetic tree of *R. flavipes* hindgut prokaryotic content (from Fisher, *et al.*, 2007).

Recent research on environmental bacteria has revealed that many uncultivated bacteria belong to new phylogenetic clades, and 16sRNA sequencing has helped in identifying these bacteria (Fisher, et al., 2007). In most cases of uncultivated bacteria arising from the hindgut of the termite, more than ninety percent of the phylotypes are novel, having no close relatives represented in the database sequences (Brune and Ohkuma, 2011). Among the phyla present within the *R. flavipes* gut, Spirochetes are the most dominant. Bacteroidetes, Firmicutes, and Endomicrobia are the second most dominant groups (Brune and Ohkuma, 2011). These groups mentioned make up approximately 80% of the bacteria in the gut community (Brune and Ohkuma, 2011). Part of the minor population in the termite gut is represented by methanogenic bacteria that have been characterized also by their 16sRNA sequences. A remarkable feature of lower termites is the various associations of the bacteria and archaea with gut flagellates (Brune and Ohkuma 2011).

1.4 Associations of Bacterial and Flagellate Communities

Nitrogen is one of the four essential elements required for biological processes because it is mandatory for protein and nucleic acid synthesis (Meuti, 2008). However, nitrogen is often limited in terrestrial ecosystems due to many factors including the nature of the carbon-nitrogen bond. The most critical factor in nitrogen restriction is the energy associated costs in transforming atmospheric nitrogen, N_2, to biologically useable forms such as NH_3. In termites, prokaryotic bacteria and

methanogenic Archaea fix nitrogen because they contain nitrogenase enzymes capable of converting N_2 to NH_3. The high energy depends associated with nitrogen fixation can be compensated for because of the intimate associations of endosymbionts with protozoan counterparts (Meuti, 2008). The oxidation of carbohydrates into pyruvate, which is later discussed, is done through aerobic respiration in protozoans. Energy released by this catabolic process can be used by methanogens. In addition, nitrogenase enzymes carried by endosymbionts require anoxic environments. The protozoans can provide this type of environment as well. Thus, termites feed on a wide variety of cellulosic materials, such as wood, leaf litter, and soil in order to meet their energy demands created by their symbionts. However, these types of materials are poor in nitrogen. Decay-free wood consumed by termites contains as little as 0.03-0.7% nitrogen (Meuti, 2008). Since termites themselves are approximately 10% nitrogen in dry weight, termites are very efficient at converting virtually unusable compounds into energy.

Associations of prokaryotes with gut flagellates are frequently observed, and as already stated; gut flagellates themselves are the hosts of prokaryotic symbionts (Noda, *et al.*, 2005). Each flagellate can harbor a dense population of intracellular endosymbionts or surface-attached ectosymbionts, or both (Brune and Ohkuma, 2011). As a result, flagellate associated prokaryotes constitute a large proportion of the gut bacterial population. Flagellate associated prokaryotes can be identified by comparing 16sRNA gene sequences of isolated flagellate cells versus prokaryotic

containing flagellates, and can also be viewed using scanning electron microscope technology. Ectosymbionts residing on the outersurface of the flagellates are often comprised of *Treponema* spirochaetes (Brune and Ohkuma, 2011). On the contrary, Bacteroidetes and Endomicrobia are often associated with flagellates as endosymbionts. In addition, methanogenic symbionts of termites are present both as endosymbionts in flagellates and as ectosymbionts attached to the hindgut surfaces (Shinzato, *et al.*, 1999).

The metabolic interactions between the prokaryotic and eukaryotic communities are essential for the acquisition of nutrients by the termite host. The intricate digestion system of termite involves the digestion of wood particles by enzymatic processes. Upon entering the hindgut, partially digested particles are phagocytosed by protozoa and lignocelluloses are broken down into polysaccharides. The polysaccharides can be further degraded to pyruvate that is transported to hydrogenosomes of members in the phylum Parabasalia (orders Hypermastigida and Trichomonadida) and fermented into acetate, CO_2, and H_2 (Stingl, 2004). On the contrary, members of the order Oxymonadida do not possess hydrogenosomes and are the most abundant protozoa in *R. flavipes*. Therefore, in this species, different fermentative processes may be at work. Ultimately, the breakdown of cellulose into D-glucose in flagellates results in glycolytic and Acetyl-CoA pathways being utilized by endosymbiotic bacteria. The common depictions of the digestive processes taking place are shown in Figure 6.

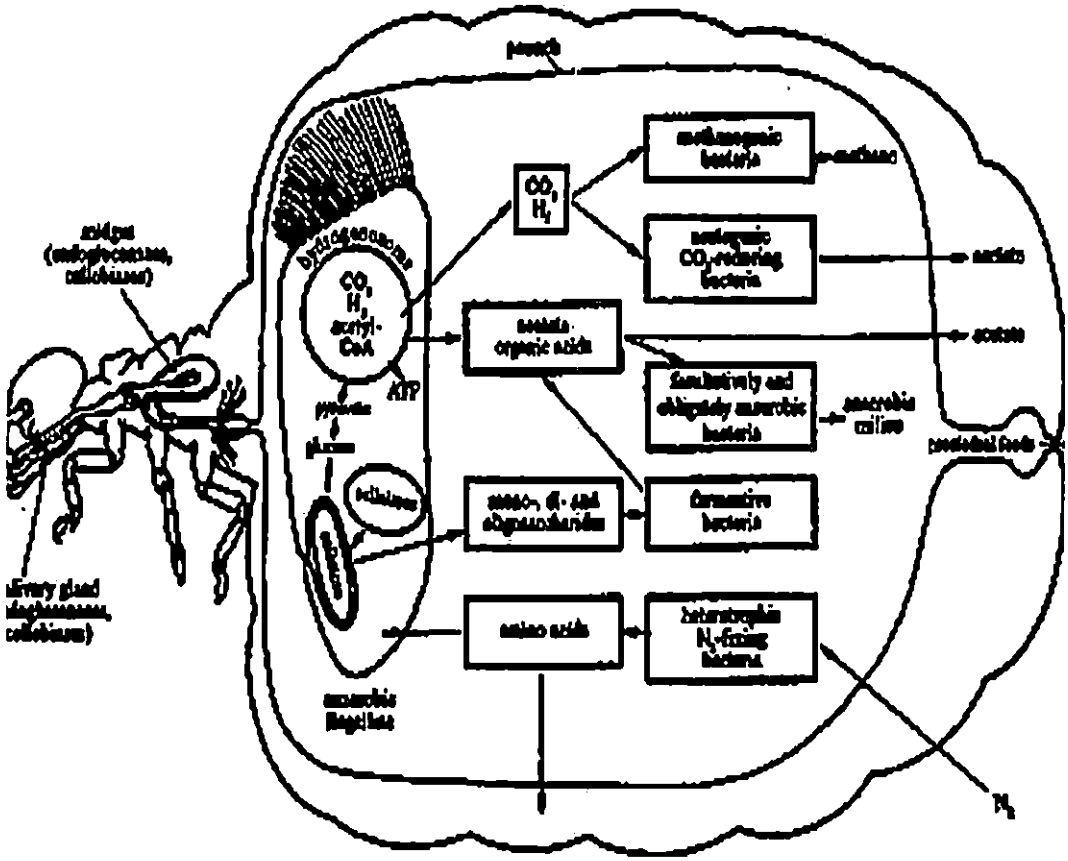


Figure 6. General metabolic pathways used by flagellate and bacterial symbionts in the termite hindgut (Radek, 1999).

Carbon dioxide and hydrogen can also be utilized by acetogenic or methanogenic bacteria to make acetate. In addition, the hydrogen consumption of methanogens is expected to promote anaerobic cellulose decomposition in the lower termite hindgut (Shinzato, *et al.*, 1999). As a result, more acetate can be sequestered as a nutrient source for the host termite. Acetate is the major solute accumulated in termites and is absorbed and utilized by the termite host to support its own respiration (Bignell, 2011).

The contribution of the diverse bacterial community in the hindgut of lower termites to the fermentative process is far from clear (Brune and Ohkuma, 2011). The bacteria associated with the flagellates clearly represent the majority of prokaryotes in the system, but there are diverse populations that are either free swimming (mostly Spirocheatal forms) or attached to the gut epithelium (Brune and Ohkuma, 2011). Figure 7 represents the collective actions of the symbiotic microflora inside the lower termites (Brune and Ohkuma, 2011).

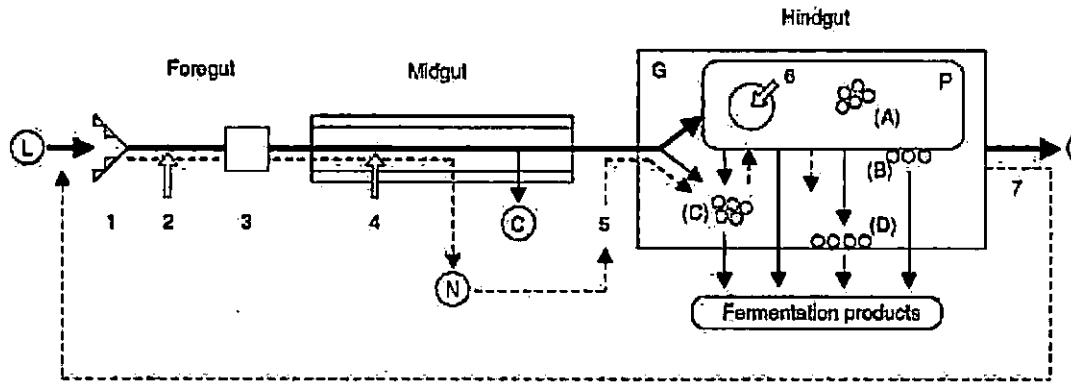


Figure 7. Symbiotic digestion in wood-feeding lower termites. Bold lines show the path of insoluble material, thin lines are soluble degradation of products that can be used by the termite, dashed lines are the recycling of nitrogen compounds, and hollow arrows show where different enzymes are secreted by the termite. Letters or numbers are as follows: (a) endosymbionts (b) ectosymbionts attached to flagellate (c) non-attached ectosymbionts (d) ectosymbionts attached to the gut wall (L) lignocellulose (C) carbohydrates (N) nitrogenous compounds (F) fecal matter (L) gut lumen (P) protozoa or flagellates (1) mandibles (2) salivary glands (3) proventriculus (4) midgut epithelium (5) Malpighian tubules (6) digestive vacuoles (7) proctodeal feeding (from Brune and Ohkuma, 2011).

1.5 Study of Eukaryotic Symbionts

Leidy first described termite flagellates from *R. flavipes* in 1877 (Lewis and Forschler, 2004). In the past seventy years, the taxonomic status of these flagellates residing in the *R. flavipes* gut have been described and revised many times (Lewis

and Forschler, 2004). It has been hypothesized that flagellate communities could be used in termite identification because distinct communities are specific to certain termites. Lewis and Forschler (2004) conducted the identification of flagellates in *R. flavipes*, as well as the number of concentrations of flagellates for each caste type. Their study showed that flagellates can actually be used in identification of separate subterranean termite species by comparing flagellate content between *R. flavipes, R. hageni,* and *R. virginicus.*

Symbiotic flagellates residing in the hindgut of subterranean lower termites play a critical role in cellulose digestion (Hu, 2008). Results from a study conducted by Xing Ping Hu (2004) showed that starvation of *R. flavipes* workers resulted in total elimination of three species of flagellates: *P. major, P. vertens*, and *T. agilis.* These results are significant in that total elimination of these cellulolytic and facultative cellulolytic species resulted in higher mortality. In these experiments, significant reduction was also noted in four other flagellate species: *D. fimbriata, D. gracilis, H. elongatum, and S. kofoidi.* Results also showed that three species of flagellates did not suffer any reduction or elimination, but instead increased in number that included: *Monocercomonas* spp., *T. trypanoides*, and *S. flagellata.* Overall, starvation of this species results in loss of important flagellate species needed in cellulose digestion and nutrient acquisition (Table 2). This study is important in understanding which species are most vulnerable to defaunation under variable conditions.

Table 2. Protist population abundance in differing feeding treatments of *R. flavipes* (Hu, 2008).

Species	Fresh field-collected	40-day feeding on filter paper	40-day starvation
Dinenympha fimbriata	2,050 ± 428	1300 ± 200	100 ± 120
D. gracilis	1,100 ± 216	1,000 ± 140	200 ± 50
Holomastigotes elongatum	1,700 ± 277	600 ± 100	100 ± 50
Monocercomonas sp.	900 ± 130	1,000 ± 600	2,160 ± 250
Pyrsonympha major	1,800 ± 200	700 ± 80	0
P. vertens	1,000± 400	900 ± 600	0
Spironympha flagellate	200 ± 70	300 ± 80	200 ± 60
S. kofoidi	2,200 ± 328	900 ± 100	100 ± 50
Trichomitus trypanoides	400 ± 130	600 ± 150	400 ± 80
Trichonympha agilis	3,660 ± 186	1,200 ± 555	0

1.6 Study of Prokaryotic Symbionts

In the last twenty years, research has focused primarily on the function of prokaryotic microorganisms and their interactions with their eukaryotic counterparts. As stated previously, the hindgut microbiota of termites includes an abundant and morphologically diverse population of Spirochetes (Breznak, 2002). Eutick, *et al.* (1978), was able to show that elimination of Spirochetes from the gut of the termite *Nasutitermes exitiosus* could be achieved by treatment with metronidazole that resulted in a rapid decrease in termite survival (Breznak, 2002). It was discovered later that Spirochetes found in termite guts are closely associated to the genus *Trepnoma* by comparison of known 16sRNA sequences. To confirm Eutick's work, Leadbetter (1999) was able to culture and isolate these Spirochetes species for the first time. Over time, it could be concluded that Spriochetes contribute to the carbon, nitrogen, and energy requirements of termites through acetogenesis and nitrogen

fixation and that their elimination from guts reduces the life span of termites significantly (Breznak, 2002).

Leadbetter and Breznak (1996) sought to investigate the relationship between methanogens and acetogens after 16sRNA analysis had revealed that they were both present within termite's guts, and because both rely on hydrogen as an energy source for their fermentative processes. Two methanogen strains from *R. flavipes* were isolated and named RFM-1 and RFM-2. Density of methanogens in hindguts of *R. flavipes* was found to be in large numbers (10^6 viable cells gut^{-1}). Epifluorescence microscopy revealed that these strains are also associated primarily with the hindgut wall, either attached directly or attached to other prokaryotes that are attached to the hindgut wall. These strains were found to be restricted to hydrogen and carbon as energy sources within the medium that they were grown in. It was ultimately found that in subterranean termites, methanogenesis dominates fermentative processes.

Mapping and location of specific species of bacteria helps in painting a picture of how nutrients and products are transferred between Eukaryotic and Prokaryotic symbionts. Moreover, 16sRNA sequencing has allowed both for the identification of bacteria that cannot be cultured and for the building of phylogenetic relationships of symbiotic termite species. This method has also been helpful in analyzing the effects of antibiotic treated populations of microflora versus controlled populations in termites.

1.7 Study of Heated Conditions on Termites

Temperature has a strong influence on termite foraging and seasonal activities (Harap, *et al.*, 2005). In addition, Harahap, *et al.* (2005), also determined a linear regression analyses based increase in the consumption of *R. flavipes* worker termites due to temperature and humidity in the collection field (Figure 8).

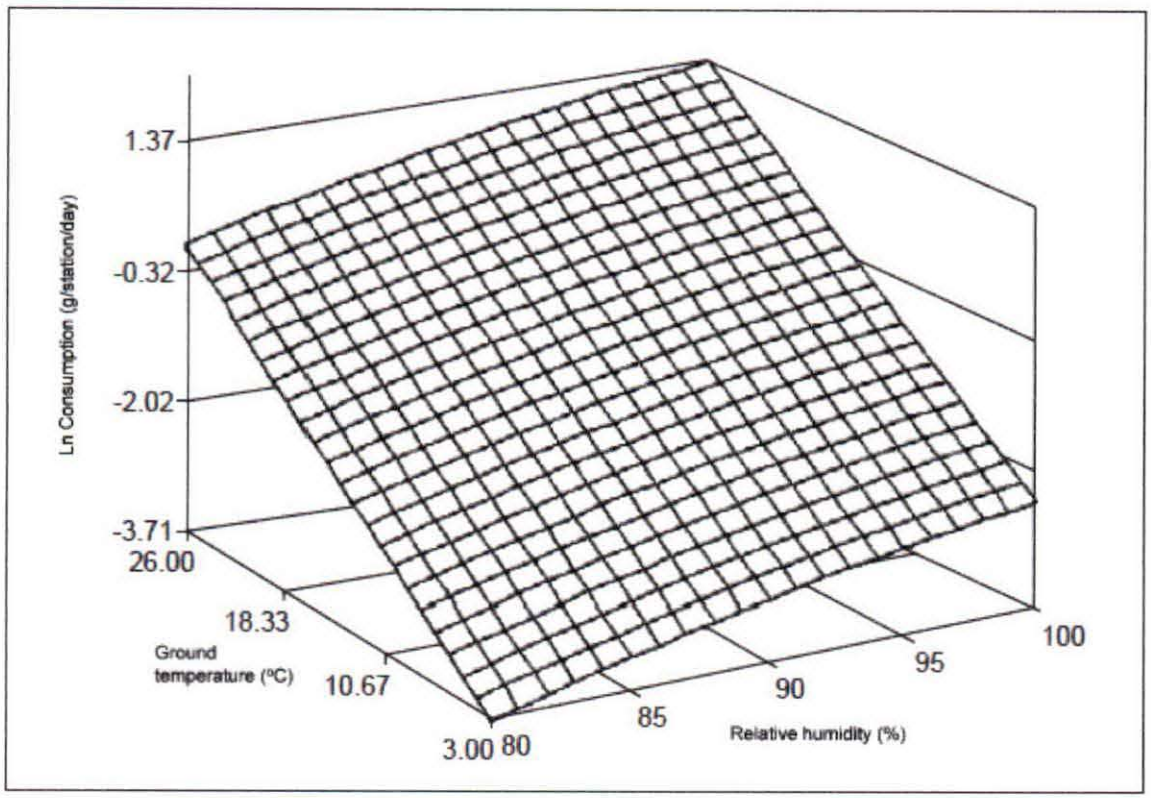


Figure 8. Linear regression model of predicted feeding response of *R. flavipes* under temperature and % humidity conditions (adapted from Harap *et al.*, 2005).

Spomer, *et al.* (2008), also showed that *R. flavipes* workers increased the rate of feeding as well as the horizontal transfer of flagellates through proctodeal feeding in vitro. These two experiments show a direct increase in feeding due to temperature.

However, in the last study mentioned, *R. flavipes* was shown to have significant mortality rates above 32^0C. This is most likely due to significant water loss and the inability to thermoregulate at higher temperatures.

1.8 Study of Antibiotic Effect on Termites

Earlier studies of antibiotic treatment on termites have used gentamicin, rifampin, and metronidazole. Research by Raina, *et al.* (2004), included the exposure of the termite *Coptotermes formosanus* to gentamicin and gentamicin plus metronidazole treatment. Only two concentrations were used (0.05% and 0.20%) to feed paired alates for three days. Paired alates were found to have a decrease in the number of produced progeny up to thirty days after removal from antibiotic feeding. Thus, antibiotic treatment led to a significant time period for recovery of nest flagellate populations needed in digestion. It was also noted that these lower concentrations did not affect feeding of the termites. This study is important because it shows the sensitivity of this species to metronidazole at lower concentrations. To date, antibiotic susceptibility of symbiotic microflora has been examined only at limited dosages that only have the intention of showing the killing effects to prokaryotic and eukaryotic communities housed within the termite gut.

First derived from aomycin produced by numerous *Streptomyces* species, metronidazole has had wide applications in treating anaerobic protozoa and bacterial infections. Metronidazole is a low molecular weight compound that diffuses across the cell membranes of anaerobic and aerobic microorganisms (Lofmark, *et al.*, 2010).

However, antimicrobial activity is limited to anaerobes. By itself, metronidazole is inactive and becomes activated through the reduction step of anaerobes. Metronidazole can be reduced by ferrodoxin in the mitochondria of obligate anaerobes. Since the compound is more favorably reduced in the low oxidation/reduction environment, ferrodoxin or flavodoxin will readily donate their electrons to metronidazole. The metronidazole ultimately captures electrons and throws the cell concentration resulting in ATP depletion of the cell. Additionally, the metronidazole radicals react rapidly with proteins, RNA, and DNA resulting in the death of the bacterium (Wouden, *et al.*, 2001). The exact mechanism of the drug has not been completely solved, but it inhibits DNA synthesis and causes DNA damage resulting in breaks of DNA strands.

The major route of elimination of metronidazole and its metabolites is the urine and some within fecal excretion. In addition, concentrations of metronidazole that increase past the MIC of anaerobes can be found in saliva and colon tissue.

There have been proposed mechanisms of resistance to metronidazole in anaerobic bacteria (Table 3). However, resistance among anaerobic pathogens is still recognized as very low and uncommon. Resistance of bacterial populations in termites has been scantly studied, although many of the previously 16S rRNA genetically sequenced bacteria have been shown to be resistant in other organisms (Roe, *et al.*, 2001; Applebaum and Chatterton, 1978; Gal and Brazier, 2004; Fiehn, 1987).

The objectives of this experiment are to determine if, and what effect metronidazole and heat have on the symbiotic microflora of *R. flavipes*. Based on past research, two hypotheses can be formed for this project: (1) Greatest decrease in overall diversity and abundance of flagellate community will be seen in populations treated with highest concentrations of metronidazole at 30^0C, followed by populations treated with highest concentrations of metronidazole at 25^oC, non-treated metronidazole populations at 30^0C, and finally controlled populations. (2) Greatest increase in mortality rate and mortality percentage will be seen in termite populations treated with metronidazole at 30^0C, followed by populations treated with metronidazole at 25^0C, non-treated metronidazole populations at 30^0C, and finally controlled populations

Chapter 2
Materials and Methods

2.1 Maintenance and Collection

Corrugated cardboard was rolled into five-inch thick the rolls and rolls were wrapped with large rubber bands. Rolls were then wetted with distilled water and place underneath rotten logs or woody debris area in the University of Kentucky Arboretum. Sites of rolls were marked with small orange-wired flags and numbered. There were over thirty sites in the Arboretum alone, and flags marked 19, 20, and 21 were used in this study (Figure 9). Coordinates for collection sites are as follows: Colony 19 (38.014569^0, -84.508064^0), Colony 20 (38.014646^0, -84.507936^0), and Colony 21(38.014539^0, -84.507866^0).

Figure 9. Location of Colony 19 (green), 20 (yellow), and 21(Red). Larger view of University Kentucky Arboretum area (top). Closer view of sampling area (bottom).

Cardboard rolls were collected approximately one week later. To ensure proper transfer, rolls were immediately placed inside of plastic bags and sealed, then placed into a plastic container and taken to the University of Kentucky Entomology Laboratory. Termites were then collected from the rolls using a shaking method while wearing Fisherbrand Nitrile gloves. Due to number of termites needed, only Colony 19 and Colony 21 used in experimentation.

For stock, collected termites were placed into plastic container (L 10 5/8" x W 7 5/8" x H 3 3/4 ") with sterilized chemical free mulch. Distilled water was used to water one side of mulch in the termite container after all termites had been removed from the cardboard. In the case that all termites could not be removed, the cardboard roll was directly placed into hole dug within the mulch inside the container, and termites left the roll on their own. Plastic containers with termites were then placed into the 25^{0}C environmental chamber set at 80% humidity with no light.

A total of fifteen termites were collected from each colony and placed into three 0.5 mL microcentrifuge tubes for Eukaryotic and Prokaryotic content comparison of starting versus treated and control colonies. Non-bleach paper towels previously cut to fit the diameter of the bottom of petri dishes were put into the bottom of sterile petri dishes. Experimental or control group termites were randomly picked off of the roll on the day of collection using sweeping or shaking action and placed into sterile petri dishes with sterilized, non-bleached paper towels. Sterile distilled water or metronidazole solution was added on one side of the petri dish to control or experimental colonies, respectively. Petri dishes containing termites were

then placed into 25^0C or 30^0C environmental chambers both set at 80% humidity with no light for two weeks. Following incubation, termites were collected and put into microcentrifuge tubes on days of death and mortality numbers were recorded. Termites were separated accordingly, and tubes were labeled for protozoan, bacterial, and mitochondrial 16sRNA species identification. Termites were checked every day for humidity inside of dish, mortality, and consumption of mulch.

2.2 Identification of Termite Species

Stock termites from colonies at sites 19, 20, and 21 all contained soldiers that were used in morphological identification. Identification of termites took place under a Nikon SMZ-18 dissecting microscope. Species determination was based on modified descriptions from Lewis and Forschler (2006). Confirmation of species identification was done using genomic sequencing by the University of Kentucky Entomology Lab.

2.3 Treatment Colonies

Two dishes from each colony were treated with 100 to 200 µL of a denoted metronidazole concentration (0.01%, 0.05%, 0.15%, 0.25%) and were placed inside of their designated environmental chamber. A total of ten worker individuals were placed into each experimental and control petri dishes. Parafilm was wrapped around the outside of the petri dish to ensure moisture stability and escape control. Sterilized forceps were used to gather deceased individuals in each petri dish during the

duration of the experiment. Deceased individuals were recorded and placed into 0.5 mL microcentrifuge tubes, labeled, and stored in -20^{0}C.

2.4 Measurement of Eukaryotic Content

Termite individuals were surface sterilized with 70% ethanol for one minute followed by two sterile water washes for one minute each. Fine tipped forceps were sterilized using 70% ethanol and Bunsen burner flame. Hindguts of termites were dissected underneath a sterilized fume hood environment and gut contents were crushed with a sterile pestle. Homogenized contents were placed into a 1.5- mL microcentrifuge tube with 1,000 µL of sterile phosphate-buffer solution (pH 7.2). Contents were centrifuged for five minutes on 12,000x *g*. Ten µL of contents were then transferred to the hemocytometer. Cells were systematically counted and flagellates were identified using shape, size, and extracellular features.

2.5 Measurement of Prokaryotic Content

Termite individuals were surface sterilized using a 70% ethanol wash for one minute followed by two sterile water washes for one minute each. Gut contents were dissected using sterilized fine-tipped forceps and crushed using a sterilized pestle. Gut cells were collected by centrifugation and were resuspended in 180 µL enzymatic lysis buffer (20mM Tris-HCL {pH 8.0], 2 mM ethylenediaminetraacetic acid {EDTA}, and 1.2% Triton X-100), mixed with lysozyme (20mg mL^{-1}), and incubated for thirty minutes at 37^{0}C. Next, 25 µL proteinase K and 200 µL buffer AL (Qiagen) were added, and the mixture was vortexed and incubated for thirty minutes at 70^{0}C.

DNA was then extracted using the Qiagen DNeasy Tissue Kit (Qiagen), starting at step 4 of the "Purification of Total DNA from Animal Tissue" protocol that accompanies the extraction kit.

The 16S rRNA genes for each DNA extraction were amplified by polymerase chain reaction (PCR) using a GeneAMP 9700 PCR System. Each 40-µl reaction contained 0.8 µL DNA extract, 1 x PCR buffer (10mM Tris-HCl, 50 mm KCl, and 0.1% Triton X-100), 2 mM $MgCl_2$, 200 µM each dNTP, 1.6 U *Taq*, 14.08 µL ddH_20, and 2 µM each primer, 41F (3'-TCGATTAAAAGATTGTATATTAT -5'{Weidner, *et al.*, 1996}) and 1389R (5'-ACGGGCGGTGTGTACAAG-3'{Marchesi, Et Al., 1998; Osborn, Et Al., 2000}).

The PCR cycling included: 2 min of initial denaturation at 95°C followed by 24 cycles at 95°C for 30 seconds, 50°C for 1 minute, and 74°C for 4 minutes, and a final elongation step at 74°C for 10 minutes. The correct PCR product size (~1.4 kb) was confirmed by electrophoresis on a 1.0% low-melting-point agarose gel. PCR cleanup was done using a QIAquick PCR Purification Kit (Qiagen) following manufacturer protocols.

2.6 Statistical Analyses

All eukaryotic content and mortality data were tested for normality using Chi-Square and Shapiro-Wilk's test for normality on ToxStat Version 3.4. In addition, F-test, ANOVA (Dunnett's Test, Bonferroni T-Test, Tukey's Test), Williams' Test, and student's t-test were used to test for statistical significance within data sets.

Correlation of metronidazole treatment was tested using single-regression analysis while heat treatment was tested using multiple regression analysis.

Chapter 3
Results

3.1 What affects do heat and antibiotic application have on the flagellate population?

Temperature fluctuations and humidity play a vital role in termite biology and behavior (Harap *et al.*, 2005). The abundance of flagellate populations residing within the hindguts of termites has been shown to determine feeding activities of the termite host. Although termites are known to produce their own cellulase, a decrease in flagellate population has been shown previously to decrease termite consumption resulting in higher mortality rates due to antibiotic application (Raina, *et al.*, 2003). Furthermore, loss of certain flagellate species in *R.* flavipes, such as *T. agilis*, has been shown to increase mortality of termites due to the inability to properly receive nutrition (Xu, *et al.*, 2010). Therefore, it was important to investigate the affects of both heat and metronidazole on the protozoan populations.

In order to measure the affect of heat on flagellate populations, flagellate population numbers for both 25^0C and 30^0C chambers were compared. It was expected that those termites abundantly feeding in the 30^0C chamber would have the highest mortality rates because of an increase in consumption of metronidazole and overall decrease in flagellate populations over time. When metronidazole-treated groups were compared to one another between the two chambers, the overall loss of protozoans differed slightly, but no significant differences could be found (Figures 10

& 11). However, control groups between the two chambers differed significantly in total protozoan population loss by Day 5 (Figure 12). The total amount of protozoa remaining within termites that died by Day 5 in the 30^{0}C chamber groups was lower compared to 25^{0}C chamber groups. These results signify the effect of heat on protozoan populations. Heat may have had an effect on flagellate populations because of bacterial overgrowth, O_2 increase within the hindgut due to increased respiration because of heated conditions, or the inability to effectively thermoregulate and retain normal hydration levels for healthy flagellate populations.

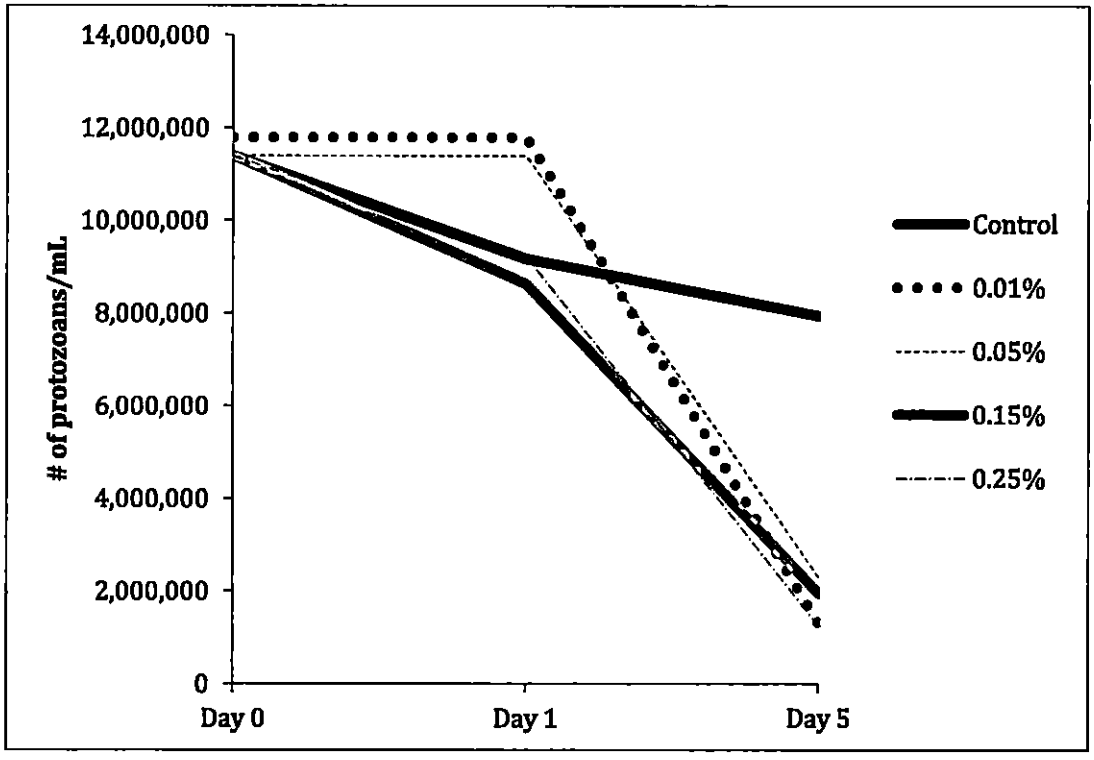


Figure 10. Protozoan depletion in 25^{0}C groups over the first five days of two-week experiment.

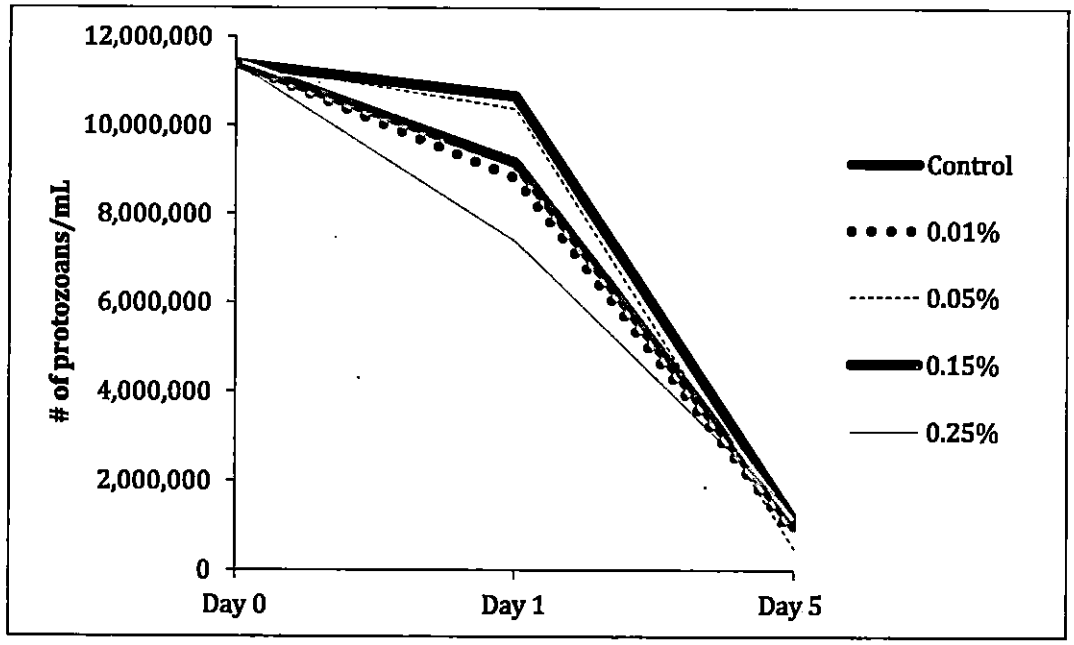


Figure 11. Protozoan depletion in 30^{0}C groups over the first five days of two-week experiment.

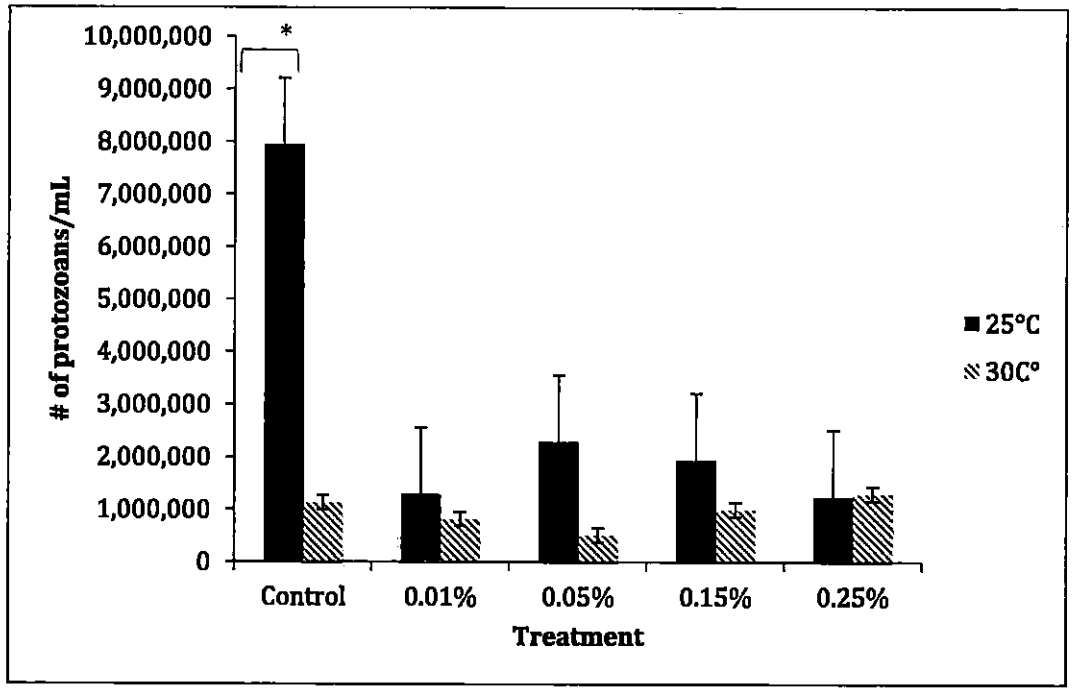


Figure 12. Protozoan depletion compared on Day 5 in 25^{0}C and 30^{0}C chambers (students t-test*p<0.05).

As expected, loss of protozoans was lower in control populations compared to those treated with metronidazole. The largest drop in flagellate populations could be seen on Day 5 of the experiment. ANOVA testing ($\propto$=0.05) was used to try and detect significant differences between control and metronidazole-treated groups on Day 5 in the 25^0C and 30^0C chambers (Figure 13 & 14) Although no significant differences were found in the 30^0C chamber, significant differences could be found between control and all experimental groups in the 25^0C chamber.

Control and 0.01% treated in 25^0C environment also showed significant differences when compared using student's t-test (α=. 05). On the contrary, 30^0C conditions killed most all populations at about the same rate regardless of treatment conditions (Figures 12 & 14). Comparisons of total protozoan population loss between both chambers for Day 5 yielded no significant values (not shown). Additionally, total loss of protozoans between two chambers did not differ significantly.

The results combined analyzing both heated and metronidazole-treated conditions signify that a threshold of concentration of metronidazole is reached in which lower concentrations have the same affect on flagellate populations as higher concentrations. This also shows that the metronidazole was working because control groups still had significantly higher values of protozoans still intact by Day 5. Furthermore, the effect of heat plus metronidazole at lowest concentrations of 0.01%, 0.05%, and 0.15% showed the greatest amount of decrease in protozoan numbers by

Day 5 in the 30^{0}C chamber. Thus, heat slightly enhances the effect of metronidazole compared to 25^{0}C and 30^{0}C control groups.

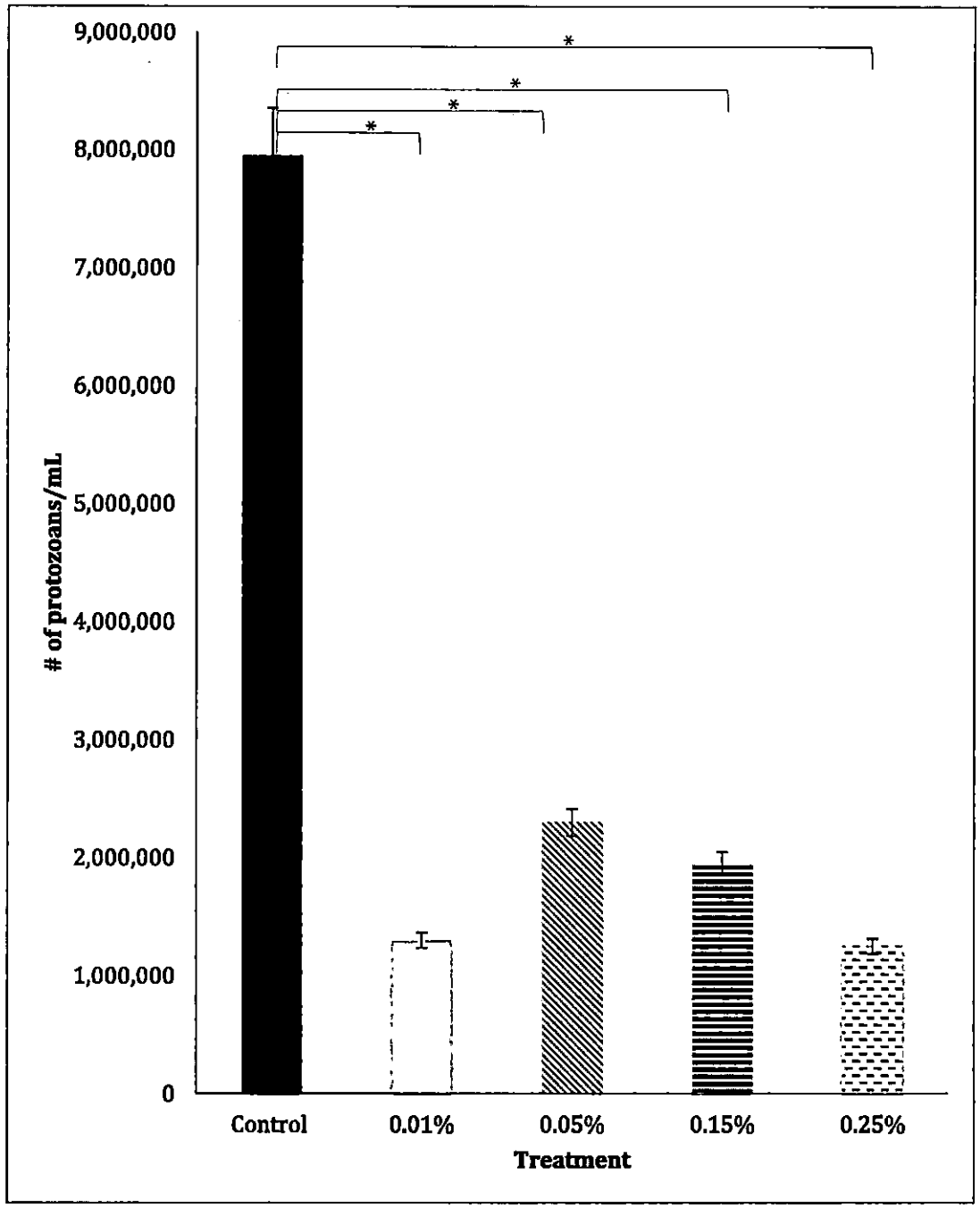


Figure 13. Total amount of protozoans in hindguts of termite 25^{0}C groups on Day 5 with standard error percentages (ANOVA*$p<0.05$).

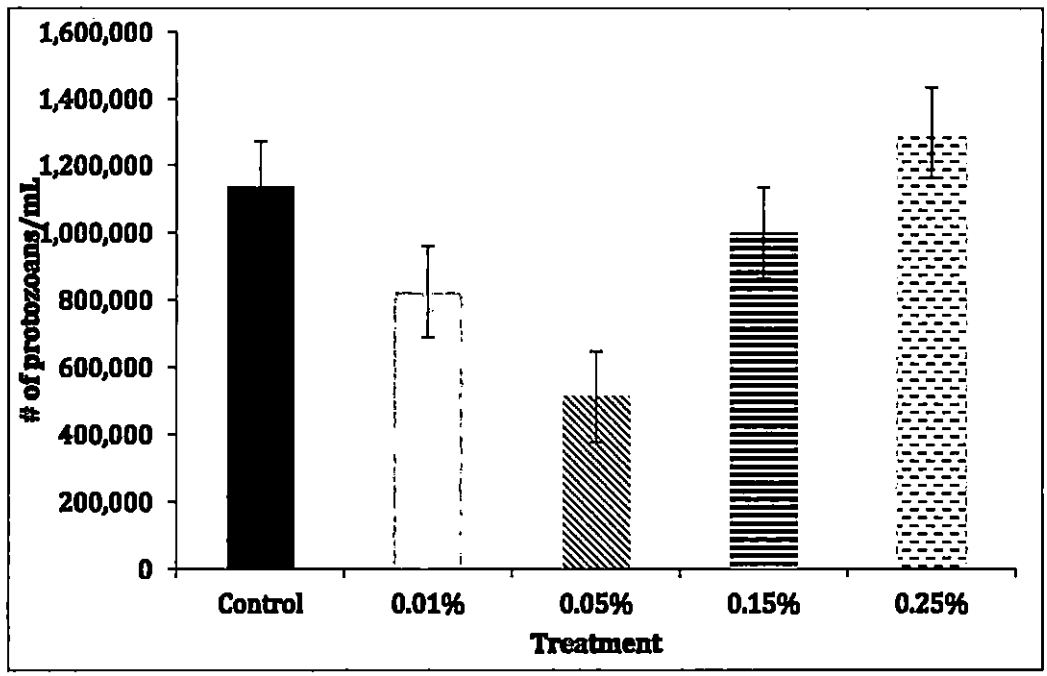


Figure 14. Total amount of protozoans in hindguts of termites in 30^{0}C groups on Day 5 with standard error percentages.

3.1 Consumption rates of termites

Because the a decrease in flagellate populations could be noted in all groups in heated or metronidazole-treated conditions, it was important to next measure the affect of decreasing flagellate populations on consumption rates. All termites from all experimental and colonies had measurable consumption that ranged from 0.02 to 0.15 grams of paper with average consumption at 0.07 grams. The fastest consumption was seen in 30^{0}C conditions, though the amount of consumption differing between metronidazole-treated control groups was not found to be significantly different. In

contrast, the 25^0C conditions showed inhibitory effects of consumption at higher concentrations of metronidazole (Figure 15a and b).

a.

b.

Figure 15. Visual comparison of a 30^0C consumption versus b. 25^0C consumption. Note the decreased consumption in 0.25% metronidazole treated population from the 25^0C conditions.

The combined effects of these results indicate that flagellate loss affects the total consumption. Yet, flagellate loss does not always result in a decrease in consumption as was observed in the 30^0C metronidazole-treated groups. Most interesting was the behavior noted in the 30^0C chamber compared to the 25^0C chamber. Higher concentrations of metronidazole in the 25^0C chamber had inhibitory affects possibly because of the ability of the termites to sense the antibiotic within the filter paper, as inhibition of consumption was not seen in the same concentrations of metronidazole noted in the 30^0C chamber.

3.3 How does consumption affect mortality?

Even though consumption varied between 30^0C and 25^0C groups due to behavior, the effect of flagellate loss in normal 25^0C chamber conditions led to a decrease in consumption and higher mortality in termites. In order to determine what affect the loss of flagellate populations had on termite survival, survivorship and final total mortality of termites were compared between groups. All control and experimental groups began with forty total termites. Both control and experimental groups in the 25^0C had termites survive until the end of the experiment (Figure 16). In contrast, termites in the 30^0C environmental chamber all died by Day 10 (Figure 17). The behavior in feeding of termites in the 30^0C chamber caused a more rapid decline in survivorship because of the increased consumption of metronidazole. It was also found using student's t-test ($\propto$=0.05) that control groups between the two chambers significantly differed in terms of total mortality (Figure 18). Other groups showed

little or no difference in terms of mortality rates leading to the conclusion that metronidazole had similar effects on treatment groups. Lastly, two-tailed t-test revealed that significant differences could be noted between total mortality in 25^0C and 30^0C environmental chambers (Figure 19).

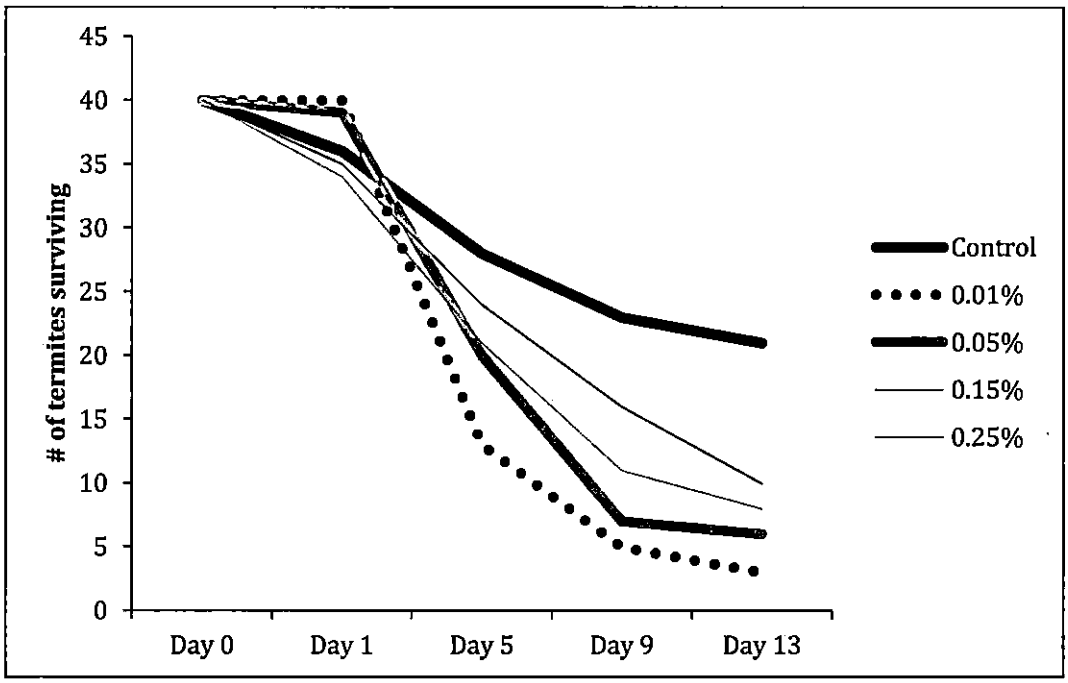


Figure 16. Survivorship curves associated with 25^0C chamber groups.

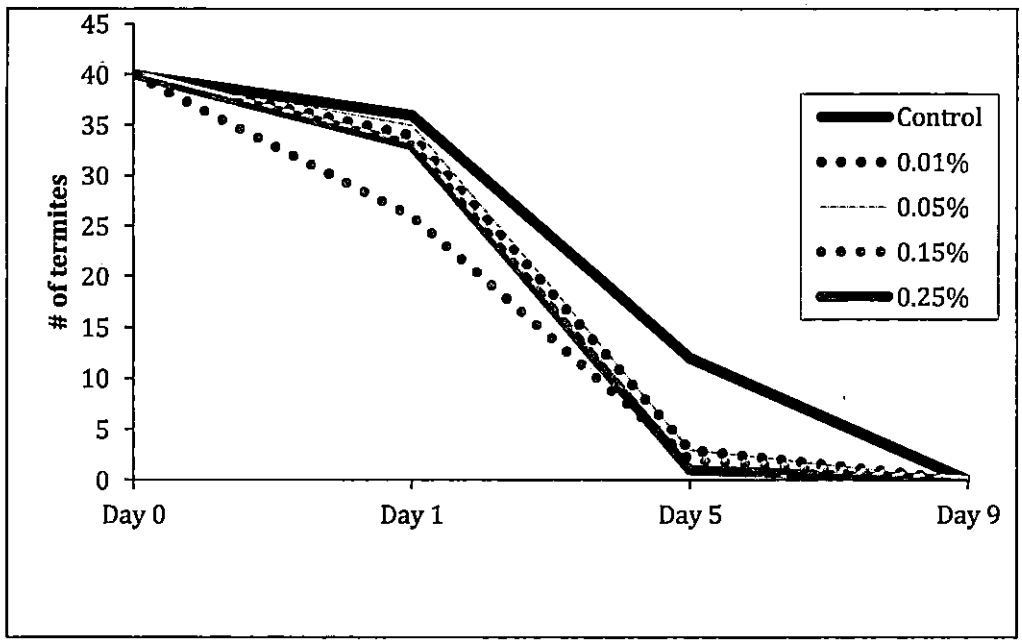


Figure 17. Survivorship curves associated with 30^0C chamber groups.

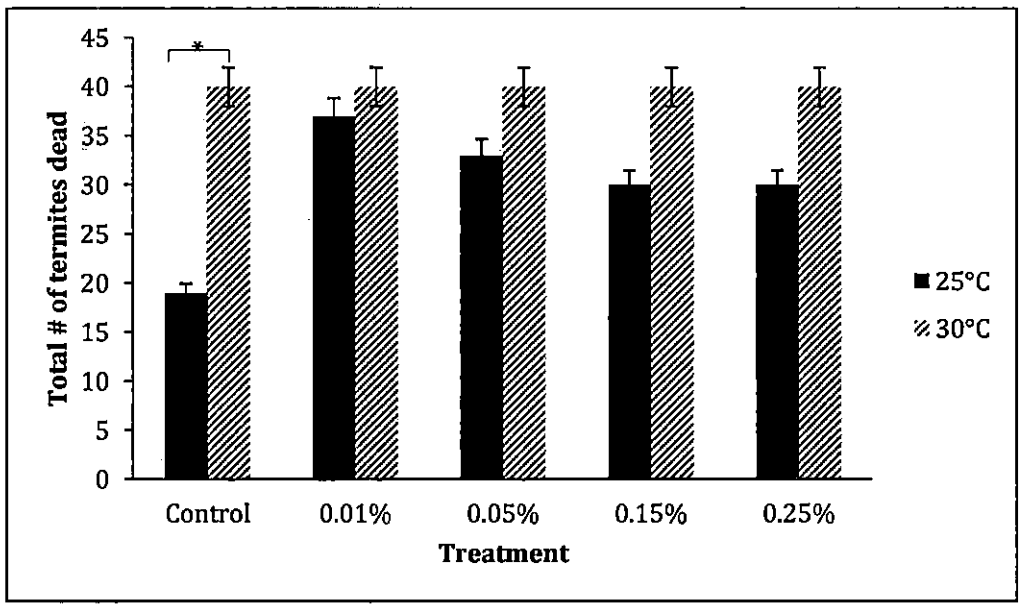


Figure 18. Total mortality between groups at different temperatures with standard error percentages (student's t-test*$p<0.05$).

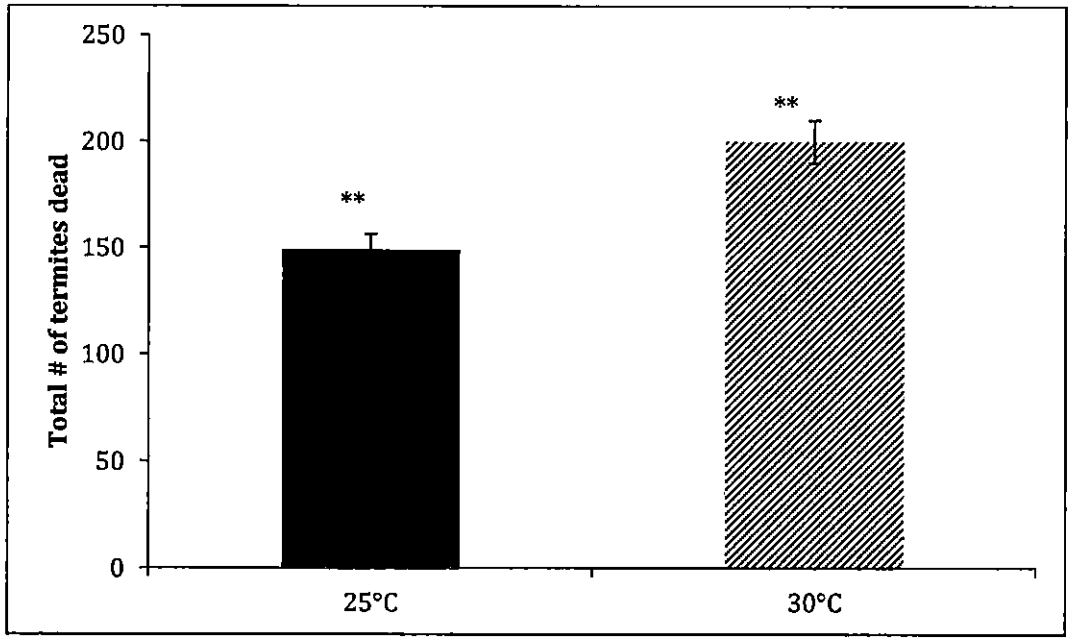


Figure 19. Total mortality between two environmental chambers with standard error percentages (two tailed t-test**$p<0.01$).

Lastly, because consumption decreased when concentration of metronidazole increased, the mortality was compared between groups of each chamber. The total mortality per day was tested and compared in control and experimental groups of both chambers. All in all, day 5 was the time of peak of mortality in all termite groups in terms of death per day (Figure 20 and 23). The highest mortality occurred in 25^0C 0.01% metronidazole treated groups on Day 5 of treatment (Figure 20). In the 25^0C environmental chamber, ANOVA and Tukey's test ($\propto$=0.05) revealed significant differences between control group mortality and mortality for 0.01% and 0.05% metronidazole treatment groups on day 5 (Figure 20). All other groups did not differ significantly from the control group in the 25^0C chamber. There was also not a significant difference between the 0.01% treatment group mortality and other

treatment group mortality for that day. Concentration treatments of 0.01% and 0.05% had large effects on mortality rates between Day 1 and Day 5 in the 25^{0}C chamber (Figure 21).

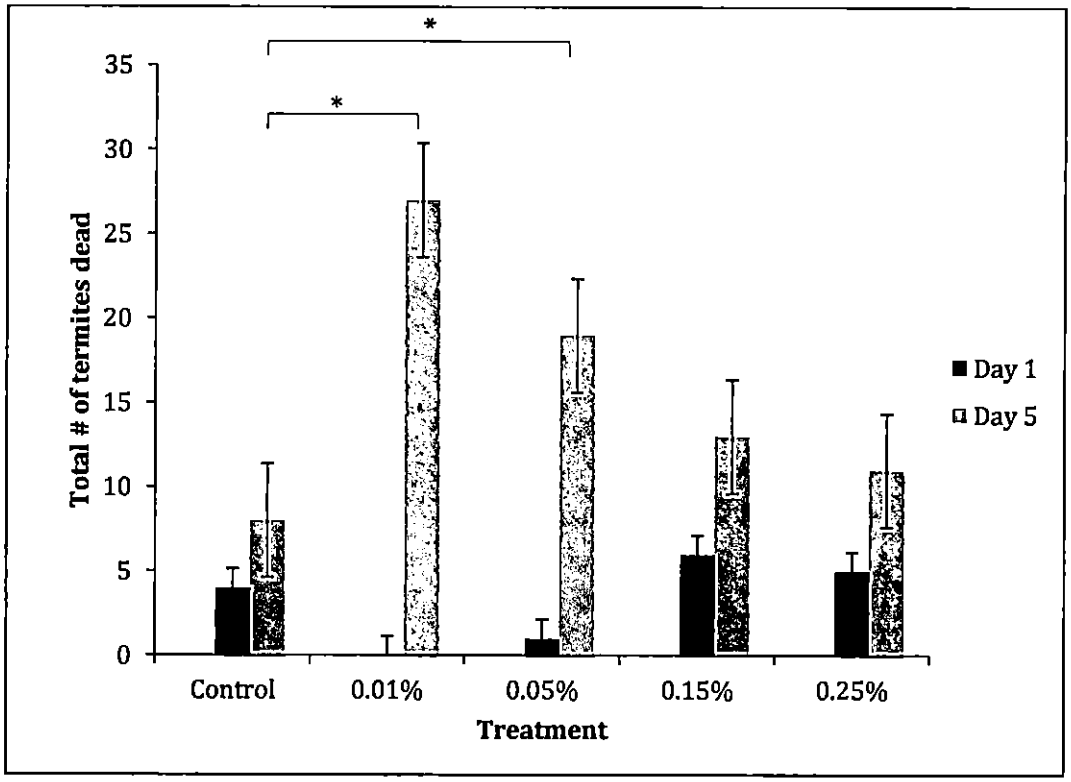


Figure 20. 25^{0}C chamber total mortality per day with standard error percentages on Day 1 and Day 5 (ANOVA*$p<0.05$).

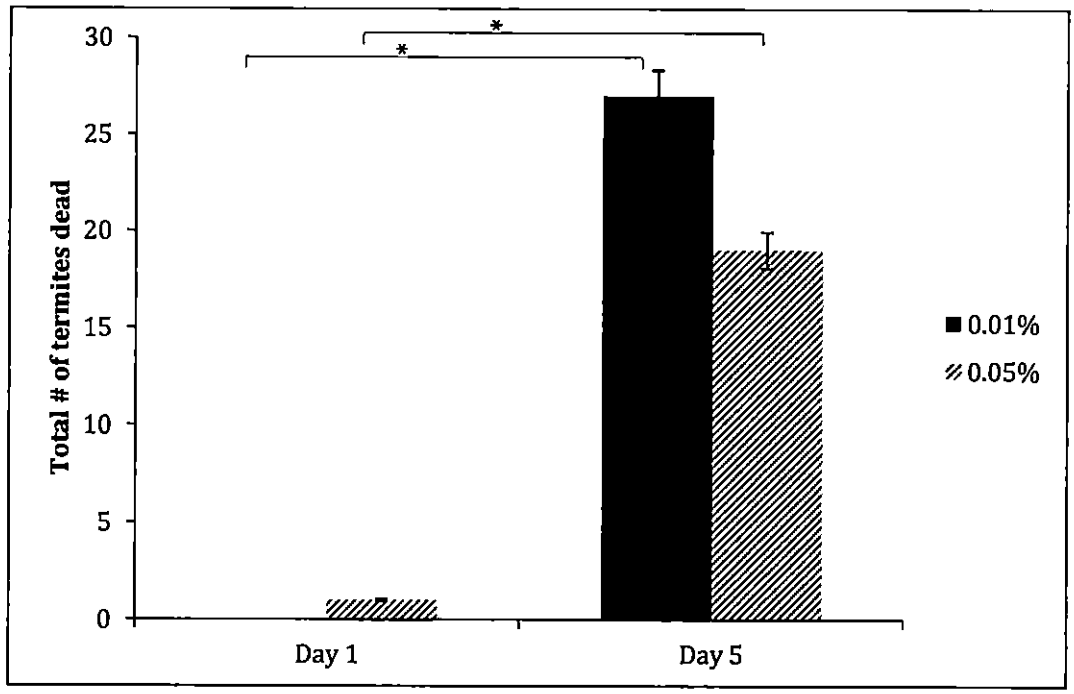


Figure 21. Mortality between Day 1 and Day 5 0.01% and 0.05% treatment groups in 25^{0}C chamber with standard error percentages (student's t-test*p<0.05).

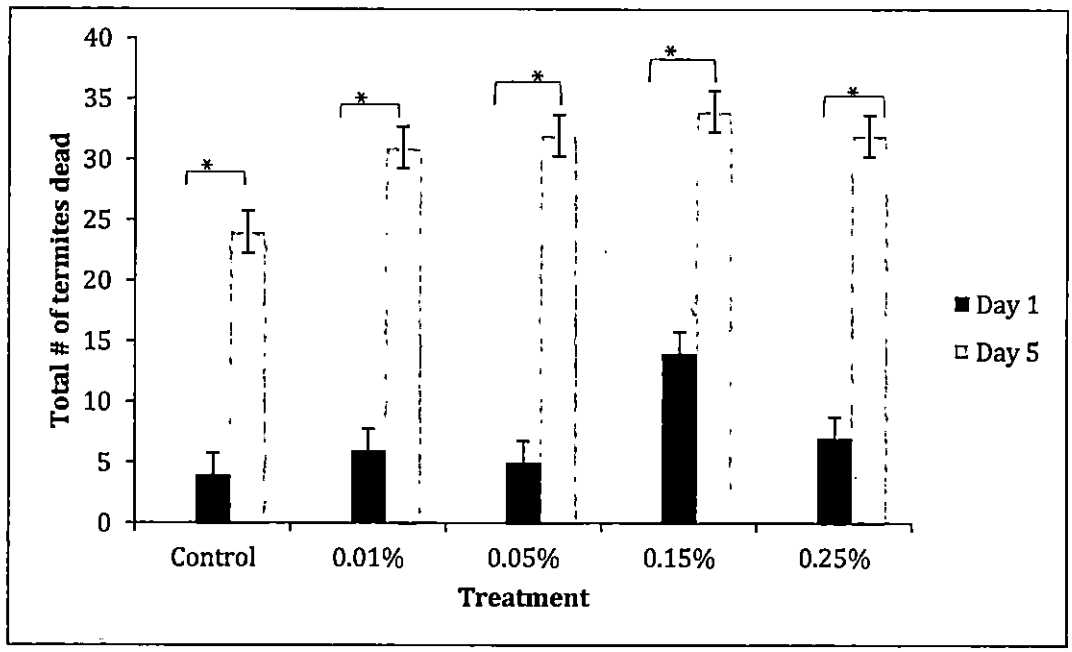


Figure 22. 30^{0}C chamber total mortality per day with standard error percentages on Day 1 and Day 5 (student's t-test*p<0.05).

Significant difference in total deaths per day between day 1 and day 5 in 30^0C control and metronidazole-treated groups that was similar to the 25^0C noted differences in 0.01% and 0.05% concentrations (Figure 22). Furthermore, significant differences between 0.05%, 0.15%, and 0.25% treatment groups and control group for Day 5 was found using Williams Test ($\propto$=0.05) (Figure 23).

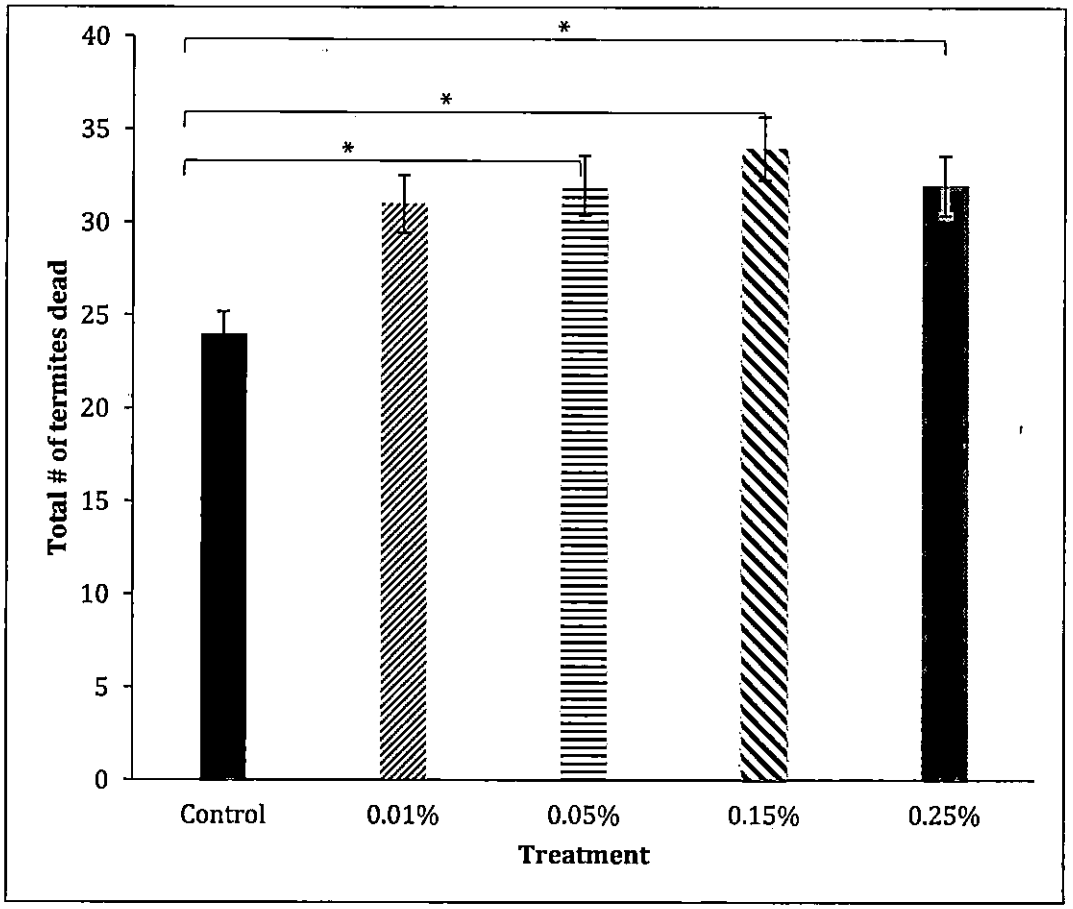


Figure 23. Mortality in 30^0C chamber on Day 5 of experiment with standard error percentages (Williams test*p<0.05).

Single linear regression analysis revealed that metronidazole plays a direct role in mortality as well as protozoan depletion. Additionally, multiple

regression analyses correlated heat application to higher mortality and protozoan depletion.

Although metronidazole and heat act synergistically to contribute to higher mortality and protozoan loss, it can be established that higher concentrations of metronidazole do not have any increased affect on mortality at higher or normal temperatures. Likewise, the lowest concentration of metronidazole was shown to have similar effects in mortality as well as contributing to a decrease in flagellate populations in both chambers.

Chapter 4

Discussion

Workers within a termite nest not only comprise the majority of a colony, but also contribute to vital jobs for sustainability of that colony. Most importantly, it is the worker caste that performs the proctodeal feeding that is shared by members of a group. This type of behavior is used as an efficient delivery system to acquire microflora contents beneficial to the whole colony. The increased feeding activities of *R. flavipes* in heated conditions has been demonstrated before, and it was found that moisture had a direct impact on termite survival (Green, *et al.*, 2005; Harap, *et al.*, 2005). This study confirms these findings, as termites needed at least 80% humidity conditions in chambers in order to prevent desiccation and mortality. Possibly due to proctodeal feeding, increased mortality was shared by all groups in 30^0C chambers. An increase in proctodeal feeding regimen may have been the result of overeating activity observed in these groups. Interestingly, the effect of higher concentrations of metronidazole in the 25^0C chamber showed a decrease in feeding activity compared to the constant feeding of groups in the 30^0C with no inhibition. Yet 25^0C groups had only slightly higher population numbers of protozoans by day 5, the critical turning point during the experiment in which mortality and flagellate loss peaked in both groups. An explanation for this observation could be the result of less energy needed in 25^0C groups compared to the 30^0C groups for vital functions: in 30^0C groups, perhaps the demand for energy outweighed the inhibition to eat based

on the sensing of metronidazole. This would explain the higher mortality numbers also seen in 30^0C groups.

The affect of metronidazole on gut microflora has been studied in other insects previously, and results showed in no significant effects on cellulase production in the midgut (Treves and Martin, 1994). Alternatively, the consequences of defaunation of protozoan populations could cause severe effects for cellulase production in the hindgut region. Although the result of metronidazole has been studied on the rate of reproduction in *R. flavipes* alates, the effect of this antibiotic had not yet been studied in worker populations.

The sensitivity of termite microflora to this antibiotic stem from the mechanism of action exhibited by metronidazole. Since the drug is favorably reduced in low oxidation/reduction conditions present in the *R. flavipes* hindgut, it can be deduced that it would have drastic effects on anaerobic microflora. Results from this study confirm previous studies involving metronidazole and its role in increasing mortality in treated populations.

Previous research has also shown that metronidazole has had most effect on ectosymbiont species of prokaryotes. More specifically, many ectosymbionts have been identified as methanogens, and destruction of these populations would lead to decreased nutrient acquisition by termites resulting in mortality. Although the confirmation of prokaryotic and eukaryotic species was not done in this study, the evidence gathered suggests that metronidazole had the most effect of ectosymbionts species of prokaryotes. An interesting result in this study was the threshold reached in

termite mortality, regardless of the concentration of metronidazole. Past studies have failed to recognize the effects of varying concentrations of metronidazole. This study is the first to show that extremely low concentrations of metronidazole have effective mortality responses in treated termites.

Experiments conducted within this study successfully addressed proposed objectives in determining the affects of heat and metronidazole on the symbiotic microflora of *R. flavipes*. Furthermore, it can be established that heat or metronidazole both work to deplete protozoan populations in termites, and in combination have synergistic effects on termite mortality. I am very confident in the results presented within this experiment because they not only reflect past research findings, but also offer new details to expand upon in further research.

Both the prokaryotic and eukaryotic populations could not be identified for this experiment as originally proposed. More so, the specific identity of flagellate species was not done because of time constraints. Identification of protozoans and bacterial symbionts could have led to more conclusive results in determining those species that are most important in nutrient acquisition for the termite, and if any populations are resistant to metronidazole treatment leading to better identification of genera already found to inhabit the *R. flavipes* gut. This experiment could have been improved upon had genomic sequencing taken place. Likewise, a larger sample size and longer duration of experimentation were lacking do to budgetary constraints. However, preserved termite specimens from this experiment can still be used for sequencing and identification in further studies conducted at Morehead State

University, and sequencing identity could shed new light on specific causes of mortality in treated groups found in this study.

Future studies should be completed to determine if lower concentrations of antibiotic are effective in causing significant mortality. In relation to this study, research concerning other aspects of antibiotic application to termite reproduction, nutrient acquisition by measurement of nitrogen fixation, and evolutionary patterns in symbiotic bacteria could lead to a better understanding of the sustained complex relationships exhibited by termites worldwide. Lastly, minimal work has focused on antibiotic application due to fears of antibiotic resistance arising in pest control. Although, as previously indicated, antibiotic research can span much further than only pest control application. With the advent of quantitative PCR and microarray technology, the determination of which genes in termites with differing diets can offer new focus to those symbiotic microflora most efficient in energy production. The impact of this research could lead to innovative biofuel development through gene manipulation, or even waste management by the engineering of novel bacteria.

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